Regulation of the *Bacillus subtilis ytmI* Operon, Involved in Sulfur Metabolism

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The YtlI regulator of *Bacillus subtilis* activates the transcription of the *ytmI* operon encoding an L-cystine ABC transporter, a riboflavin kinase, and proteins of unknown function. The expression of the *ytlI* gene and the *ytmI* operon was high with methionine and reduced with sulfate. Using deletions and site-directed mutagenesis, a *cis*-acting DNA sequence important for YtlI-dependent regulation was identified upstream from the −35 box of *ytmI*. Gel mobility shift assays confirmed that YtlI specifically interacted with this sequence. The replacement of the sulfur-regulated *ytlI* promoter by the *xylA* promoter led to constitutive expression of a *ytmI'-lacZ* fusion in a *ytlI* mutant, suggesting that the repression of *ytmI* expression by sulfate was mainly at the level of YtlI synthesis. We further showed that the YrzC regulator negatively controlled *ytlI* expression while this repressor also acted on *ytmI* expression via YtlI. The cascade of regulation observed in *B. subtilis* is conserved in *Listeria* spp. Both a YtlI-like regulator and a *ytmI*-type operon are present in *Listeria* spp. Indeed, the Lmo2352 protein from *Listeria monocytogenes* was able to replace YtlI for the activation of *ytmI* expression and a lmo2352'-*lacZ* fusion was repressed in the presence of sulfate via YrzC in *B. subtilis*. A common motif, AT(A/T)ATTCCTAT, was found in the promoter region of the *ytlI* and lmo2352 genes. Deletion of part of this motif or the introduction of point mutations in this sequence confirmed its involvement in *ytlI* regulation.

All living organisms require sulfur for the synthesis of proteins and essential cofactors. Sulfur can be assimilated either from inorganic sources, such as sulfate and thiosulfate, or from organic sources, such as sulfate esters, sulfamates, sulfonates, cysteine, methionine, or their derivatives. In *Bacillus subtilis*, sulfate is transported into the cell via a sulfate permease, CysP, related to inorganic phosphate transporters (24). Sulfate is subsequently reduced to sulfide probably in four steps involving ATP sulfurylase, adenosine 5′-phosphosulfate (APS) kinase, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) reductase, and sulfite reductase (3, 25, 45). An *O*-acetyl-L-serine thiol-lyase, the *cysK* gene product, condenses sulfide and *O*-acetylserine (OAS) to form cysteine (45). Sulfonates, cysteine, and methionine can be used as sulfur sources by *B. subtilis*. Several alkanesulfonates are taken up by a sulfonate ABC (ATP-binding cassette) transporter and then converted to sulfite by a $FMNH_2$-dependent monooxygenase (46). The transport of L-cystine, the oxidized form of cysteine, has recently been investigated. Three systems are present in *B. subtilis*: two ABC transporters, TcyABC and TcyJKLMN, and a symporter, TcyP (5). The TcyJKLMN and TcyP uptake systems are high-affinity transporters with apparent K_m values for L-cystine of 2.5 μM and 0.6 μM, respectively. In addition, the TcyJKLMN system is involved in the uptake of cystathionine, *S*-methylcysteine, djenkolic acid, and other sulfur compounds (5, 39). The *tcyJKLMN* genes belong to a large operon (operon *ytmI*),

which also encodes a riboflavin kinase, two putative flavin-dependent monooxygenases, a putative acetyltransferase, and a putative amidohydrolase (7, 39, 41, 42). The expression of the *ytmI* operon and the *tcyP* gene is regulated in response to sulfur availability, while the level of expression of the *tcyABC* operon remains low in all the conditions tested (5). In addition, the expression of the *ytmI* operon is induced by disulfide stress or paraquat (21, 29).

In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, most of the genes involved in sulfate and sulfonate assimilation, cysteine biosynthesis, and L-cystine transport are coordinately regulated in the cysteine regulon (18). The high-level expression of these genes requires CysB, a LysR-type transcriptional activator, the inducer *N*-acetylserine, and limitation in sulfur availability (18, 19). The interaction of CysB with the inducer allows it to interact with the activation sites present upstream of the −35 promoter region of the positively regulated *cys* genes (15, 19, 28). Among the genes regulated by CysB, one encodes Cbl, a second LysR-type regulator that controls the expression of the *ssu* and *tau* operons involved in aliphatic sulfonate and taurine assimilation. In *E. coli*, the MetJ repressor and a third LysR-type regulator, MetR, regulate the methionine biosynthetic pathway (12).

In *B. subtilis*, the S-box transcription antitermination system is involved in the control of expression of genes participating in methionine uptake, biosynthesis, and recycling in response to methionine availability (1, 13, 26, 38). In addition, two LysR-type regulators, CysL and YtlI, play a role in the regulation of sulfur metabolism. CysL positively controls the expression of the *cysJI* operon encoding the sulfite reductase by binding to its promoter region (14). YtlI is a positive regulator of the *ytmI* operon encoding the TcyJKLMN L-cystine ABC transporter

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TABLE 1. Bacterial strains used in this study[a]

Strain	Genotype	Source
168	*trpC2*	Laboratory stock
BSIP1214	*trpC2 ytlI::aphA3*	Reference 7
BSIP1215	*trpC2 amyE::pΔFytlI'-lacZ cat*	pDIA5575 → 168
BSIP1224	*trpC2 ytlI::aphA3 amyE::pΔFytlI'-lacZ cat*	pDIA5575 → BSIP1214
BSIP1256	*trpC2 amyE::pΔAytmI'-lacZ cat*	pDIA5599 → 168
BSIP1257	*trpC2 amyE::pΔCytmI'-lacZ cat*	pDIA5600 → 168
BSIP1258	*trpC2 amyE::pΔDytmI'-lacZ cat*	pDIA5601 → 168
BSIP1259	*trpC2 amyE::pΔGytlI'-lacZ cat*	pDIA5602 → 168
BSIP1261	*trpC2 amyE::pΔJytlI'-lacZ cat*	pDIA5606 → 168
BSIP1264	*trpC2 amyE::pΔIytlI'-lacZ cat*	pDIA5607 → 168
BSIP1519	*trpC2 ytlI::aphA3 amyE::pΔAytmI-lacZ cat*	pDIA5599 → BSIP1214
BSIP1521	*trpC2 ytlI::aphA3 amyE::pΔCytmI'-lacZ cat*	pDIA5600 → BSIP1214
BSIP1523	*trpC2 ytlI::aphA3 amyE::pΔDytmI'-lacZ cat*	pDIA5601 → BSIP1214
BSIP1589	*trpC2 amyE::pΔHytlI'-lacZ cat*	pDIA5648 → 168
BSIP1712	*trpC2 amyE::pΔBytmI'-lacZ cat*	pDIA5689 → 168
BSIP1722	*trpC2 ytlI::aphA3 amyE::pΔBytmI'-lacZ cat*	BSIP1214 → BSIP1712
BSIP1724	*trpC2 cysH::Tn10 amyE::pΔFytlI'-lacZ cat*	BSIP1175 → BSIP1215
BSIP1725	*trpC2 ΔcysJI::aphA3 amyE::pΔFytlI'-lacZ cat*	BSIP1206 → BSIP1215
BSIP1726	*trpC2 cysK::spc amyE::pΔFytlI'-lacZ cat*	BSIP1304 → BSIP1215
BSIP1731	*trpC2 amyE::pΔA(−73 A→G)ytmI'-lacZ cat*	pDIA5699 → 168
BSIP1733	*trpC2 amyE::pΔA(−65 T→G)ytmI'-lacZ cat*	pDIA5701 → 168
BSIP1735	*trpC2 amyE::pΔA(−61 G→T)ytmI'-lacZ cat*	pDIA5703 → 168
BSIP1737	*trpC2 amyE::pΔA(−49 T→G)ytmI'-lacZ cat*	pDIA5705 → 168
BSIP1738	*trpC2 amyE::pΔA(−45 T→G)ytmI'-lacZ cat*	pDIA5706 → 168
BSIP1746	*trpC2 amyE::pΔA(−50 T→G)ytmI'-lacZ cat*	pDIA5710 → 168
BSIP1754	*trpC2 amyE::plmo2352'-lacZ cat*	pDIA5717 → 168
BSIP1760	*trpC2 amyE::pΔA(−72 T→G)ytmI'-lacZ cat*	pDIA5719 → 168
BSIP1762	*trpC2 amyE::pΔEytmI'-lacZ cat*	pDIA5721 → 168
BSIP1764	*trpC2 ytlI:aphA3 amyE::pΔEytmI'-lacZ cat*	BSIP1214 → BSIP1762
BSIP1767	*trpC2 amyE::pΔA(−71 T→G)ytmI'-lacZ cat*	pDIA5727 → 168
BSIP1768	*trpC2 amyE::pΔA(−66 T→G)ytmI'-lacZ cat*	pDIA5728 → 168
BSIP1770	*trpC2 amyE::pΔA(−42 T→G)ytmI'-lacZ cat*	pDIA5730 → 168
BSIP1773	*trpC2 ytlI:aphA3 amyE::pΔA(−73 A→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1731
BSIP1774	*trpC2 ytlI:aphA3 amyE::pΔA(−65 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1733
BSIP1775	*trpC2 ytlI:aphA3 amyE::pΔA(−61 G→T)ytmI'-lacZ cat*	BSIP1214 → BSIP1735
BSIP1776	*trpC2 ytlI:aphA3 amyE::pΔA(−49 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1737
BSIP1777	*trpC2 ytlI:aphA3 amyE::pΔA(−45 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1738
BSIP1778	*trpC2 ytlI:aphA3 amyE::pΔA(−50 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1746
BSIP1779	*trpC2 ytlI:aphA3 amyE::pΔA(−72 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1760
BSIP1780	*trpC2 ytlI:aphA3 amyE::pΔA(−71 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1767
BSIP1781	*trpC2 ytlI:aphA3 amyE::pΔA(−66 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1768
BSIP1782	*trpC2 ytlI:aphA3 amyE::pΔA(−42 T→G)ytmI'-lacZ cat*	BSIP1214 → BSIP1770
BSIP1783	*trpC2 ytlI::aphA3 thrC::pxylA-ytlI spc amyE::pΔEytmI'-lacZ cat*	pDIA5679 → BSIP1764
BSIP1784	*trpC2 ytlI::aphA3 thrC::pxylA-lmo2352 spc amyE::pΔEytmI'-lacZ cat*	pDIA5687 → BSIP1764
BSIP1793	*trpC2 ΔyrzC amyE::pΔFytlI'-lacZ cat*	Materials and methods
BSIP1797	*trpC2 ΔyrzC amyE::pΔAytmI-lacZ cat*	Materials and methods
BSIP1798	*trpC2 ΔyrzC amyE::lacZ aphA3*	pAC7 → BSIP1793
BSIP1801	*trpC2 ΔyrzC amyE::plmo2352'-lacZ cat*	pDIA5717 → BSIP1798
BSIP1811	*trpC2 ΔyrzC ytlI::aphA3 amyE::pΔAytmI'-lacZ cat*	BSIP1214 → BSIP1797

[a] Arrows indicate construction by transformation; *cat* is the pC194 chloramphenicol acetyl transferase gene, *aphA3* is the *Enterococcus faecalis* kanamycin resistance gene, *erm* is an erythromycin resistance gene, and *spc* is a spectinomycin resistance gene.

(5, 7). The expression of both the *ytmI* and *ytlI* genes is high in the presence of methionine and low in the presence of sulfate as sulfur source (1, 7). This indicates the existence of a cascade of regulation for the *ytmI* operon. In this work, we analyzed the complex regulation of *ytmI* expression by the YtlI activator.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. subtilis* strains and plasmids are listed in Table 1. *B. subtilis* was grown in SP medium or in minimal medium (6 mM K_2HPO_4, 4.4 mM KH_2PO_4, 0.3 mM trisodium citrate, 5 mM $MgCl_2$, 0.5% glucose, 50 mg liter^{-1} L-tryptophan, 22 mg liter^{-1} ferric ammonium citrate, 0.1% L-glutamine) containing 1 mM K_2SO_4, 1 mM L-methionine, or 0.5 mM L-cysteine as sole sulfur source. OAS was added to the final concentration of 1 mM.

Antibiotics were added to the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 5 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}; and kanamycin, 5 μg ml^{-1}. Solid media were prepared by addition of 20 g liter^{-1} Noble agar (Difco). Standard procedures were used to transform *E. coli* (33) and *B. subtilis* (20).

The loss of amylase activity was detected as previously described (43). β-Galactosidase-specific activity was measured as described by Miller (27) with cell extracts obtained by lysozyme treatment. Protein concentrations were determined by the method of Bradford (4). One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol min^{-1} of *O*-nitrophenol at 28°C. The mean values of at least three independent experiments are presented. Standard deviations are less than 20% of the mean.

DNA manipulations. Plasmids from *E. coli* and chromosomal DNA from *B. subtilis* were prepared according to standard procedures. Restriction enzymes and phage T4 DNA ligase were used as specified by the manufacturers. DNA fragments used for cloning experiments were prepared by PCR using a High
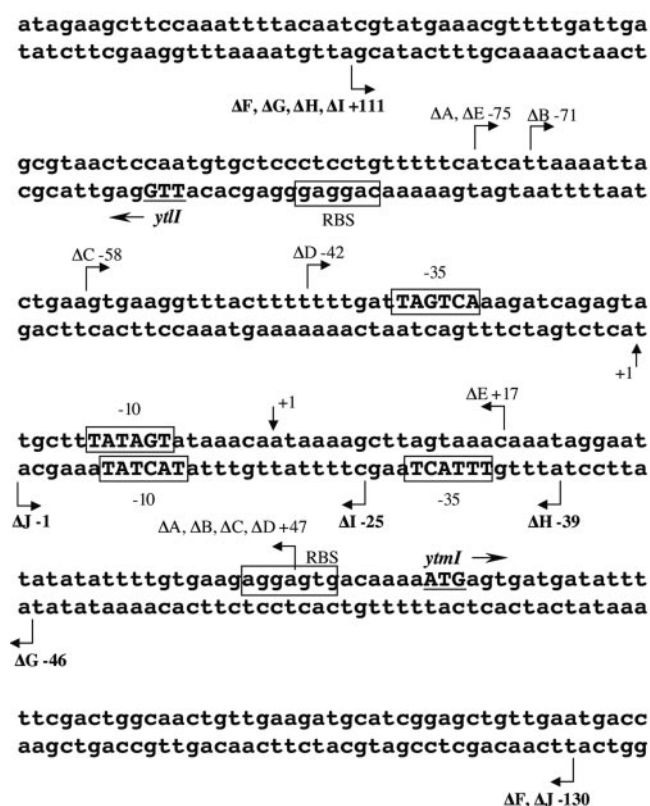
```
atagaagcttccaaattttacaatcgtatgaaacgttttgattga
tatcttcgaaggtttaaaatgttagcatactttgcaaaactaact
                         ΔF, ΔG, ΔH, ΔI +111      ΔA, ΔE -75   ΔB -71

gcgtaactccaatgtgctccctcctgtttttcatcattaaaatta
cgcattgagGTTacacgaggaggacaaaaagtagtaatttttaat
       ←  ytlI              RBS
    ΔC -58           ΔD -42              -35

ctgaagtgaaggtttactttttttgatTAGTCAaagatcagagta
gacttcacttccaaatgaaaaaaactaatcagtttctagtctcat
                                              +1
     -10         +1                  ΔE +17

tgcttTATAGTataaacaataaaagcttagtaaacaaataggaat
acgaaTATCATatttgttattttcgaaTCATTTgtttatcctta
     -10                     -35
ΔJ -1       ΔA, ΔB, ΔC, ΔD +47    ΔI -25      ΔH -39
                RBS          ytmI →

tatatatttgtgaagaggagtgacaaaaATGagtgatgatattt
atatataaaacacttctcctcactgtttttactcactactataaa
ΔG -46

ttcgactggcaactgttgaagatgcatcggagctgttgaatgacc
aagctgaccgttgacaacttctacgtagcctcgacaacttactgg
                                   ΔF, ΔJ -130
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FIG. 1. The intergenic region between the *ytlI* gene and the *ytmI* operon. The transcription start points of the *ytlI* and the *ytmI* genes (+1) are indicated by vertical arrows. The −35 and −10 regions for each gene are in uppercase and boxed. The ribosome-binding sites (RBS) are boxed, while the translational start sites of *ytlI* and *ytmI* are indicated. These two genes are transcribed divergently. The molecular elements corresponding to the *ytmI* and *ytlI* promoter regions were located on the upper and lower strand, respectively. Deletion end points of the different fusions with the *lacZ* gene are indicated by broken arrows and numbered with respect to the transcriptional start sites of the *ytlI* and *ytmI* genes, respectively.

Fidelity PCR system (Roche). Amplified fragments were purified with a QIA-quick PCR purification kit (QIAGEN).

Plasmid construction. Plasmid pAC6 (43) was used to construct transcriptional fusions between a series of deletions of the *ytmI* promoter region and the promoterless *lacZ* gene. The regions pΔA(−75 to +47), pΔB(−71 to +47), pΔC(−58 to +47), pΔD(−42 to +47), and pΔE(−75 to +17) were amplified by PCR with the creation of 5′-EcoRI and 3′-BamHI sites (Fig. 1). PCR products were inserted into pAC6 to give plasmids pDIA5599 (pΔA), pDIA5689 (pΔB), pDIA5600 (pΔC), pDIA5601 (pΔD), and pDIA5721 (pΔE), respectively. The different fusions were integrated at the *amyE* locus of strains 168 or BSIP1214 (*ytlI::aphA3*) (Table 1).

Transcriptional fusions between a series of 5′ and 3′ deletions of the *ytlI* promoter region and the promotorless *lacZ* gene were also constructed. The pΔF(−130 to +111), pΔG(−46 to +111), pΔH(−39 to +111), pΔI(−25 to +111), and pΔJ(−130 to −1) regions were amplified by PCR with the creation of EcoRI and BamHI sites (Fig. 1). The PCR products were inserted into pAC6 to give pDIA5575 (pΔF), pDIA5602 (pΔG), pDIA5648 (pΔH), pDIA5607 (pΔI), and pDIA5606 (pΔJ), respectively. The fusions were integrated at the *amyE* locus of strain 168 (Table 1).

A transcriptional lmo2352′-*lacZ* fusion was constructed as follows. An EcoRI-BamHI DNA fragment corresponding to the lmo2352 promoter region (nucleotides −266 to +47 relative to the translational start site) was generated by PCR using chromosomal DNA from *Listeria monocytogenes* strain EGD-e as template. This fragment was cloned into pAC6. The resulting plasmid pDIA5717 was integrated at the *amyE* locus of *B. subtilis* 168.

Plasmid pXT (31) was used to express the *ytlI* and lmo2352 genes under the control of the xylose-inducible promoter (p*xylA*). The complete coding sequences of *ytlI* and lmo2352 (nucleotides −45 to +953 or −64 to +958 relative to the translational start site, respectively) were amplified by PCR with the creation of EcoRI and BamHI sites. The amplified fragments were inserted into the BamHI and EcoRI sites of pXT, producing pDIA5632 (*ytlI*) and pDIA5687 (lmo2352). The *ytlI* and lmo2352 genes were then integrated by a double-crossover event at the *thrC* locus (Table 1).

Construction of the *yrzC* mutant and derivative strains. To disrupt the *yrzC* gene, a two-step PCR procedure was used. DNA fragments corresponding to the 5′ end (nucleotides −1199 to +33 relative to the translational start site) or the 3′ end (nucleotides −32 to +1140 relative to the translational stop site) of the *yrzC* gene were first amplified by PCR with a 30-bp overlapping fragment at one end of each PCR. A second PCR using the *yrzC* upstream and downstream regions and the two external primers allowed amplification of a single PCR fragment containing an in-frame deletion of *yrzC*. A truncated YrzC protein containing the first 11 amino acids of YrzC, an insertion of 10 amino acids, and the last 10 amino acids of YrzC was obtained. The wild-type strain was then cotransformed with the Δ*yrzC* PCR fragment and chromosomal DNA from either the BSIP1215 (*amyE*::pΔF*ytlI*′-*lacZ cat*) or the BSIP1256 (*amyE*::pΔA*ytmI*′-*lacZ cat*) strain. Cm^r transformants carrying these fusions at the *amyE* locus were obtained on SP plates containing 5 μg ml^{-1} chloramphenicol and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside). Blue transformants resulting from the deletion of the *yrzC* gene were obtained, giving rise to strains BSIP1793 (*ytlI*) and BSIP1797 (*ytmI*), respectively (Table 1). The deletion of the central part of the *yrzC* gene was checked by PCR, while the absence of mutation in the adjacent genes (*yrvN* and *yrvO*) was verified by sequencing the corresponding DNA fragments.

The replacement of the pΔF*ytlI*-*lacZ* by a kanamycine resistance gene at the *amyE* locus was performed by transformation with linearized pAC7 (carrying the *aphA3* gene between two *amyE* fragments), giving strain BSIP1798 (Table 1). A lmo2352-*lacZ* fusion was then introduced into this strain by transformation with linearized pDIA5717 and selection for chloramphenicol resistance.

Site-directed mutagenesis of the *ytlI* and the *ytmI* promoter regions. Plasmids pDIA5575 [pΔF(−130 to +111)*ytlI*′-*lacZ*] or pDIA5599 [pΔA(−75 to +47)*ytmI*′-*lacZ*] were used to perform site-directed mutagenesis with a Quikchange site-directed mutagenesis Kit (Stratagene). Two synthetic oligonucleotides (35 to 40 bp) complementary to opposite strands and containing the mutation were used to amplify plasmid pDIA5575 or pDIA5599. The methylated parental DNA templates were digested by the DpnI endonuclease. DNA of pDIA5575 or pDIA5599 derivative plasmids containing the point mutations was extracted. The presence of the mutation was verified by sequencing the *ytlI* or *ytmI* promoter regions. The linearized plasmids were used to transform *B. subtilis* 168. The *ytmI*′-*lacZ* fusions containing point mutations were also introduced into the *ytlI* mutant, BSIP1214. The strains obtained were listed in Tables 1 and 3.

RNA isolation and analysis. Total RNA was isolated from *B. subtilis* 168 grown in minimal medium containing 1 mM methionine. Exponentially growing cells (optical density at 600 nm, 0.8) were harvested. One gram of 0.1-mm-diameter glass beads (Sigma) was added. The cells were broken by shaking in a Fastprep apparatus (Bio101) two times for 30 s. The Trizol reagent (Gibco-BRL) was used to extract total RNA. For the primer extension experiments, the oligonucleotides IV184 (5′-TCGTATGAAACGTTTTGATTGAGCGTAACTCC-3′) (*ytlI*) and IV185 (5′-CTCCGATGCATCTTCAACAGTTGCCAGTCG-3′) (*ytmI*) were labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and hybridized with 10 μg of RNA. The primers were extended by use of reverse transcriptase, and the products were analyzed as previously described (33). The same primer was used for the generation of a sequence ladder using the method of Sanger et al. (34).

Overproduction and purification of YtlI. The *ytlI* gene (nucleotides +1 to +923 relative to the translational start site) was amplified by PCR using primers introducing a 5′-NdeI and a 3′-XhoI site and was cloned in the pET20b$^+$ vector (Novagen) digested by NdeI and XhoI. A translational fusion adding six carboxy-terminal histidine residues to YtlI was obtained. The plasmid, pDIA5621 (pET20b$^+$*ytlI*), was transformed into the *E. coli* BL21(DE3) strain (Novagen), which contains pDIA17 (30) encoding the *lacI* repressor. The resulting strain was grown at room temperature in Luria-Bertani medium to an optical density at 600 nm of 2.5. Isopropyl β-D-thiogalactoside (1 mM) was added to induce the expression of *ytlI*, and incubation was pursued for 3 h. Cells were centrifuged and resuspended in buffer I (50 mM sodium phosphate [pH 8], 300 mM NaCl, 20 mM imidazole). *E. coli* crude extracts were loaded on a Ni-nitrilotriacetic agarose column. The column was washed and the YtlI-His$_6$ was eluted with an immidazole gradient (30 to 500 mM). The purification of YtlI-His$_6$ was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).
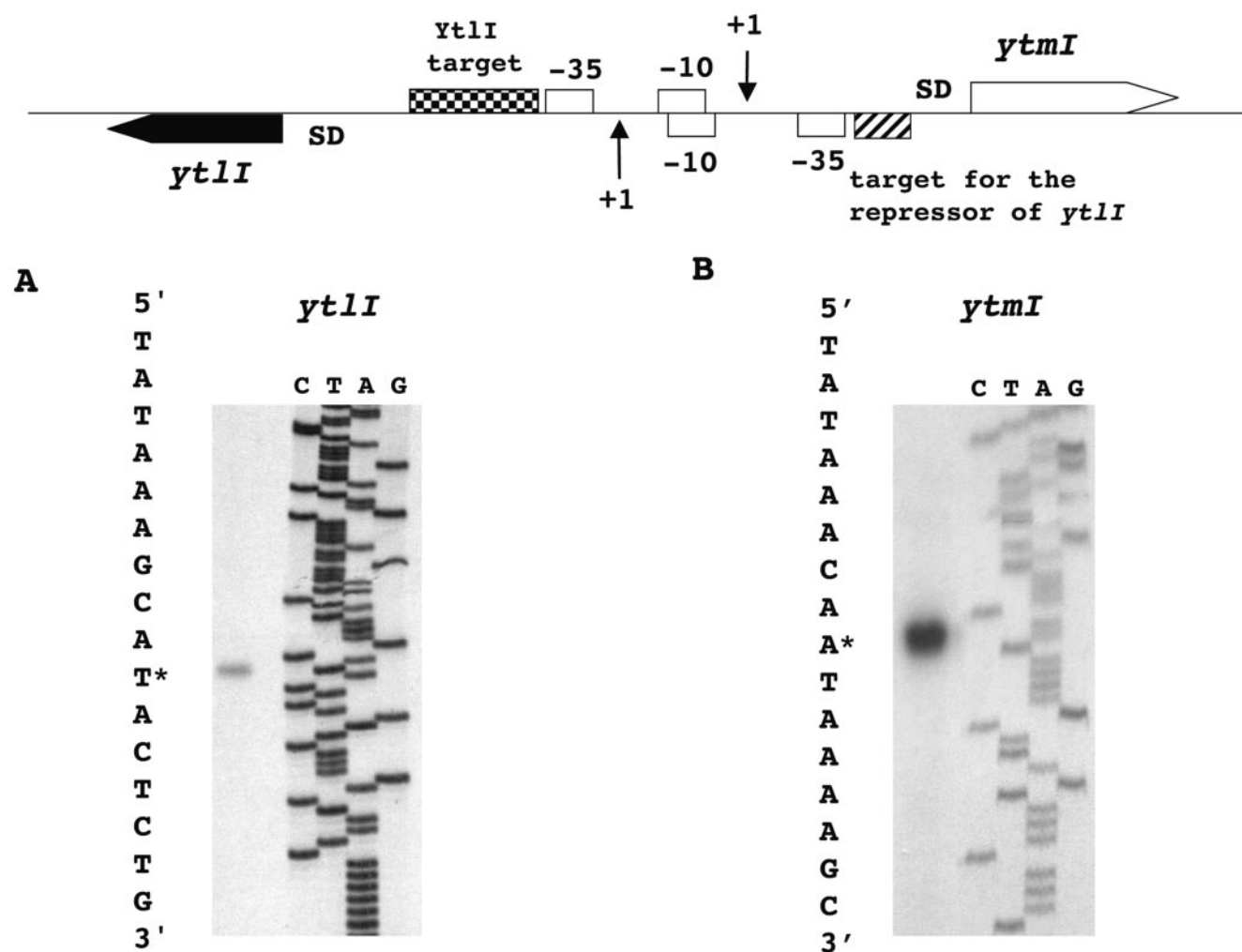
FIG. 2. Mapping of the transcription start site of the *ytlI* gene and of the *ytmI* operon by primer extension. Total RNA was extracted from *B. subtilis* strain 168 grown in minimal medium in the presence of 1 mM methionine as sole sulfur source. Primer extension experiments were performed using (A) oligonucleotide IV184, which hybridized with the *ytlI* gene, and (B) oligonucleotide IV185, which hybridized with the *ytmI* gene. For each experiment, the labeled oligonucleotide was loaded as a control. Sequencing reactions (lanes C, T, A, and G) were performed with the IV185 or IV184 oligonucleotides as primers and pDIA5599 (*ytmI*) or pDIA5575 (*ytlI*) as templates. Asterisks indicate the 5′ ends of mRNAs. SD, Shine-Dalgarno.

Gel mobility shift assays. DNA fragments containing various *ytmI* promoter regions (ΔA, −75 to +47; ΔB, −71 to +47; and ΔA containing a point mutation, A-73G or T-49G) were amplified by PCR using pDIA5599, pDIA5689, pDIA5699, or pDIA5705, respectively, as template. In these plasmids, the *ytmI* promoter region was flanked by the *cat* gene in the 5′ end and by the *lacZ* gene in the 3′ end. PCR products were obtained using [γ-^{32}P]ATP 5′ end-labeled primers located in these two flanking genes. Protein-DNA complexes were formed in 10-μl volumes, by incubating the ^{32}P-labeled DNA fragments with different amounts of YtlI-His$_6$ purified protein in binding buffer (25 mM phosphate buffer [pH 7], 2 mM MgSO$_4$, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol) in the presence of 1 μg ml$^{−1}$ of poly(dIdC). The DNA binding reaction was incubated at room temperature for 20 min. Samples were separated on a 6% polyacrylamide native gel in Tris-borate-EDTA buffer at 10 V cm$^{−1}$ for 1 h. Dried gels were analyzed with a Storm Imager (Molecular Dynamics). Radioactive signal intensities were quantified with the PDQuest software (PDI; Bio-Rad).

RESULTS

Identification of the *ytlI* and *ytmI* promoters. The DNA sequence of the *ytlI*-*ytmI* intergenic region is presented in Fig.

1. The translation initiation codons of *ytlI* and *ytmI* are a TTG and an ATG preceded by a ribosome-binding site, respectively. The 5′ ends of the *ytlI* and *ytmI* transcripts were identified by primer extension analysis using total RNA extracted from a wild-type strain grown in minimal medium in the presence of methionine (Fig. 2). Transcription was initiated at a single A located 57 bp upstream from the translational start point of *ytmI*. The deduced −35 (TAGTCA) and −10 (TATAGT) boxes of the promoter corresponded to the consensus sequence of σ^A-dependent promoters (Fig. 1). A T located 78 bp upstream from the TTG was the transcriptional start site of *ytlI*. The −35 (TTTACT) and −10 (TACTAT) regions of the promoter are quite similar to the consensus of σ^A-dependent promoters (Fig. 1).

Characterization of a *cis*-acting target sequence upstream from the *ytmI* operon. In order to identify DNA sequences necessary for the sulfur-dependent regulation of the *ytmI*

TABLE 2. Effect of the sulfur source and of the YtlI and YrzC regulators on the expression of different $ytmI'$-$lacZ$ transcriptional fusions[a]

Relevant genotype	β-Galactosidase activity (nmol ONP min⁻¹ mg of protein⁻¹)			
	$ytlI^+$		$ytlI::aphA3$	
	Methionine	Sulfate	Methionine	Sulfate
pΔA(−75 to +47)$ytmI'$-$lacZ$	535	0.9	6.5	0.7
pΔB(−71 to +47)$ytmI'$-$lacZ$	40	0.8	6.5	1
pΔC(−58 to +47)$ytmI'$-$lacZ$	20	0.9	8	0.9
pΔD(−42 to +47)$ytmI'$-$lacZ$	4	0.6	4	0.6
pΔE(−75 to +17)$ytmI'$-$lacZ$	520	6.5	4.5	4.2
pΔE(−75 to +17)$ytmI'$-$lacZ$ pxylA ytlI	ND	ND	510	530
pΔE(−75 to +17)$ytmI'$-$lacZ$ pxylA lmo2352	ND	ND	1,365	1,350
pΔA(−75 to +47)$ytmI'$-$lacZ$	535	0.9	6.5	0.7
pΔA(−75 to +47)$ytmI'$-$lacZ$ ΔyrzC	790	730	6	6

[a] Cells were grown in minimal medium in the presence of sulfate or methionine at 1 mM final concentration. The β-galactosidase activities were obtained from cultures in mid-exponential growth phase. The mean values of at least three independent experiments are presented. Standard deviations are less than 20% of the mean. For the strains containing the $pxylA$ $ytlI$ at the $thrC$ locus, minimal medium contained fructose instead of glucose, threonine (50 mg liter⁻¹), and xylose 0.2%. ONP, O-nitrophenol. ND, not determined.

operon, $ytmI$ promoter regions containing 5′ deletions were fused to the $lacZ$ gene. The fusion end points are indicated in Fig. 1. These fusions were introduced as a single copy at the $amyE$ locus of the wild-type and $ytlI::aphA3$ strains. β-Galactosidase activity was measured in the different strains grown in minimal medium in the presence of methionine or sulfate as sole sulfur source (Table 2). The expression of the pΔA(−75 to +47)$ytmI'$-$lacZ$ fusion was increased about 550-fold when methionine was used as the sulfur source instead of sulfate (Table 2, line 1). The level of expression of the pΔB(−71 to +47), pΔC(−58 to +47), and pΔD(−42 to +47)$ytmI'$-$lacZ$ fusions in the presence of methionine was reduced compared to that of the pΔA(−75 to +47) fusion (Table 2, lines 1 to 4). The introduction of an $ytlI$ gene disruption led to a decrease of expression of the pΔA, pΔB, and pΔC$ytmI'$-$lacZ$ fusions in the presence of methionine to the level observed in the pΔD$ytmI'$-$lacZ$ fusion (Table 2, lines 1 to 4). These results indicated that the DNA fragment located between nucleotides −75 and −71 was necessary to observe a high level of regulation of the $ytmI$ operon by YtlI. Further deletions in the 5′ part of the $ytmI$ promoter region led to a gradual decrease of the YtlI-dependent control in response to the sulfur source. It is worth noting that in an $ytlI$ mutant, all these fusions conserved a low but significant repression by sulfate (Table 2, lines 1 to 4). This suggested the existence of a second regulation in response to sulfur availability independent of YtlI. To determine whether the region downstream from the $ytmI$ promoter was important for this regulation, a pΔE(−75 to +17)$ytmI'$-$lacZ$ fusion was constructed. The expression of this fusion strongly decreased during growth with sulfate compared with methionine. Moreover, the residual repression by sulfate observed with the pΔA(−75 to +47)$ytmI$-$lacZ$ fusion in a $ytlI$ background was totally abolished for the pΔE(−75 to +17) fusion (Table 2,

lines 1 and 5). This suggested that a second minor site of sulfur-dependent regulation was present downstream from the $ytmI$ promoter (Fig. 2).

Complementation of a $ytlI$ mutant by the lmo2352 gene from $L.$ $monocytogenes$. Browsing the genome sequences of A-T-rich $Firmicutes$ spp., we observed that a YtlI-like regulator was present in $L.$ $monocytogenes$ (68% identity) and in $Listeria$ $innocua$ (67% identity) (http://genolist.pasteur.fr). Interestingly, a $ytmI$-like operon is present adjacent to the gene encoding the regulator in $Listeria$ species (Fig. 3A and data not shown). The similarity between the polypeptides encoded by these operons ranges from 75% identity for YtnJ and Lmo2343 or Lin2437 to 52% identity for YtnI and Lmo2342 or Lin2436. The high level of identity between the YtlI, Lmo2352, and Lin2446 regulators suggested that the regulation was conserved, prompting us to test the ability of the lmo2352 gene to complement a $ytlI::aphA3$ mutant for the expression of a $ytmI'$-$lacZ$ fusion. The lmo2352 gene was integrated in the $thrC$ locus of strain BSIP1764 containing a pΔE$ytmI'$-$lacZ$ fusion and a $ytlI$ gene disruption. In the resulting strain BSIP1784, the lmo2352 gene was expressed under the control of the $xylA$ promoter. β-Galactosidase activity was tested after growth of strains BSIP1764 and BSIP1784 in minimal medium containing 1 mM threonine, 0.2% xylose, and methionine as sulfur source. The pΔE$ytmI'$-$lacZ$ fusion was poorly expressed in a $ytlI$ mutant, while the introduction of the lmo2352 gene in $trans$ restored the expression of this fusion (Table 2, lines 5 and 7). This result indicated that the Lmo2352 protein from $L.$ $monocytogenes$ was able to replace YtlI for the activation of transcription of the $B.$ $subtilis$ $ytmI$ operon. A common target for these regulators was therefore searched in the promoter regions of the $ytmI$, lmo2351, and lin2445 operons. The comparison of the DNA sequence upstream of these operons revealed the presence of a highly conserved motif, ATTANNATTACT GN₂GTNAN₄TTANTTTTTTTGATTAG (Fig. 4A).

Site-directed mutagenesis of a conserved motif present in the promoter regions of $ytmI$, lmo2351, and lin2445. This motif, that covered the DNA fragment located between nucleotides −75 and −71 previously identified as crucial for YtlI-dependent regulation, was likely to be involved in the binding of this activator to the $ytmI$ promoter region. To confirm this hypothesis, point mutations were introduced in the conserved sequence using site-directed mutagenesis (see Materials and Methods). The pΔA$ytmI'$-$lacZ$ fusions containing various mutations were inserted at the $amyE$ locus of a wild-type strain or a $ytlI$ mutant. The level of β-galactosidase activity in the corresponding strains was determined after growth with methionine or sulfate. The introduction of point mutations did not significantly modify the level of expression of the fusions in the presence of sulfate with β-galactosidase activities ranging from 0.5 to 1 U (mg of protein)⁻¹. In contrast, the expression of the modified pΔA$ytmI'$-$lacZ$ fusions with methionine was decreased compared to that of the wild-type fusion in a $ytlI^+$ strain (data not shown). This was expected for modifications in the binding site of an activator. The contribution of the different nucleotides in the YtlI-dependent regulation of $ytmI$ expression was then estimated. To this purpose, we calculated the ratio of the β-galactosidase activity measured in a wild-type strain and in a $ytlI$ mutant after growth with methionine (Fig. 4B). Mutations in the $ytmI$ promoter region at position −73
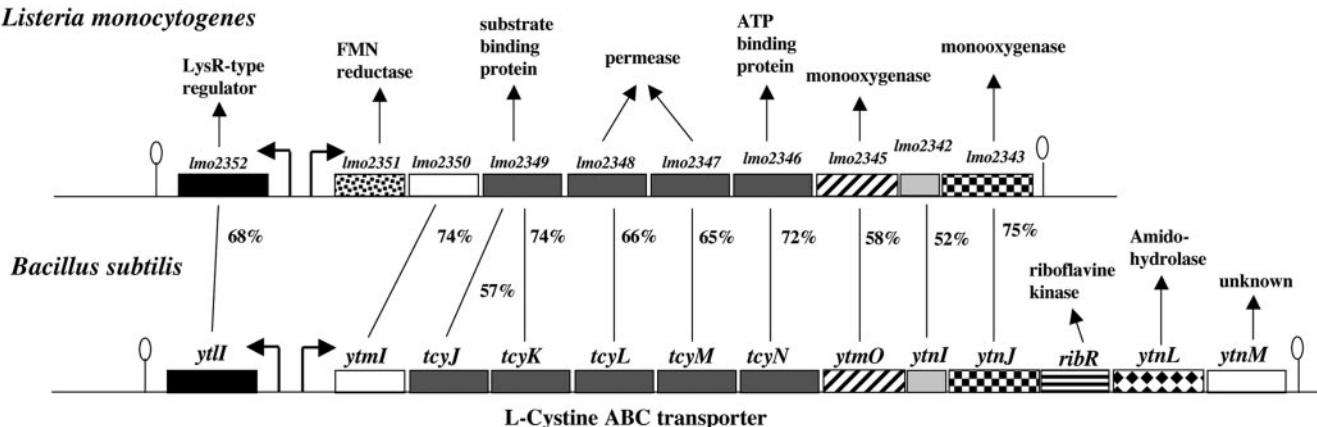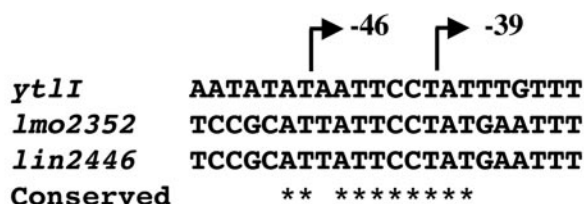
FIG. 3. The *ytlI-ytmI* locus from *B. subtilis* and *L. monocytogenes*. A: Putative transcription start sites are indicated by broken arrows, and putative terminators are represented by loops. For each gene product of the *ytmI* operon and its equivalent in the *L. monocytogenes* lmo2351 operon a percentage of identity is indicated. The genes encoding the ABC transporters are represented by dark gray boxes, the LysR-type regulators by black boxes, and the monooxygenases by crosshatched boxes (*ytmO*-like) or checkered boxes (*ytnJ*-like). B: Alignment of the promoter regions of the *ytlI*, lmo2352, and lin2446 genes. Stars indicate conserved nucleotides in the three promoters. Deletion end points of the different *ytlI'-lacZ* fusions are indicated by broken arrows.

(A→G), −72 (T→G), −50 (T→G), −49 (T→G), or −45 (T→G) had a drastic effect on the YtlI-dependent activation with a weak residual control of *ytmI* expression by YtlI compared to the wild-type promoter. An intermediate level of regulation by YtlI was observed with modifications at position −71 (T→G), −66 (T→G), −65 (T→G), −61 (G→T), or −42 (T→C).

Binding of the YtlI regulator to the *ytmI* promoter region. To determine whether YtlI controlled *ytmI* by direct binding to the promoter region of this operon, the YtlI protein fused with six histidyl residues was overproduced in *E. coli* and purified. The YtlI-His$_6$ protein was then used in gel shift DNA binding assays. A radiolabeled DNA fragment containing the wild-type ΔA *ytmI* promoter region (positions −75 to +47) was incubated with increasing amounts of YtlI. The YtlI regulator bound to this DNA fragment, forming a single complex (Fig. 5A). Gel mobility shift assays were also performed to determine more precisely the importance of some nucleotides for this binding. To this purpose, a fragment including the ΔB(−71 to +47) promoter region and two ΔA(−75 to +47) regions carrying a point mutation (A-73→G or T-49→G) were used. The binding of YtlI to the ΔB promoter region or to the ΔA(T-49→G) DNA sequence was almost undetectable (Fig. 5B and C). In gel shift experiments, the YtlI binding was more than 70-fold lower for these two promoter regions than for the wild-type ΔA DNA fragment. Furthermore, YtlI binding to the

ΔA(A-73→G) DNA fragment was decreased approximatively 15 times compared to the wild-type ΔA promoter region (data not shown). This indicated that YtlI specifically interacted with the DNA region located upstream from the *ytmI* promoter. The deletion of the DNA fragment located between positions −75 and −71 or the modification of T in position −49 virtually abolished YtlI binding, while the mutation of A in position −73 strongly reduced it. These results substantiated and extended the data obtained in vivo using *lacZ* fusions (Table 2, Fig. 4B).

YtlI-dependent regulation in response to sulfur availability. As previously shown, the expression of the *ytlI* gene was higher after growth with methionine than with sulfate (1). To determine whether the activity of YtlI as transcriptional activator was also directly modulated in response to sulfur availability, the sulfur-regulated *ytlI* promoter was replaced by the *xylA* promoter. The expression of a pΔE(−75 to +17)*ytmI'-lacZ* fusion was then tested in a wild-type strain (BSIP1762) and in a *ytlI* mutant with or without the p*xylA-ytlI* gene at the *thrC* locus (BSIP1783 and BSIP1764, respectively). The strains were grown in minimal medium containing 1 mM threonine and 0.2% xylose in the presence of methionine or sulfate. While a *ytlI* gene disruption abolished the transcriptional activation of the pΔE*ytmI'-lacZ* fusion, the insertion of p*xylA-ytlI* in the *ytlI* mutant restored the expression of this fusion after growth in the presence of methionine (Table 2, lines 1 and 6). However,
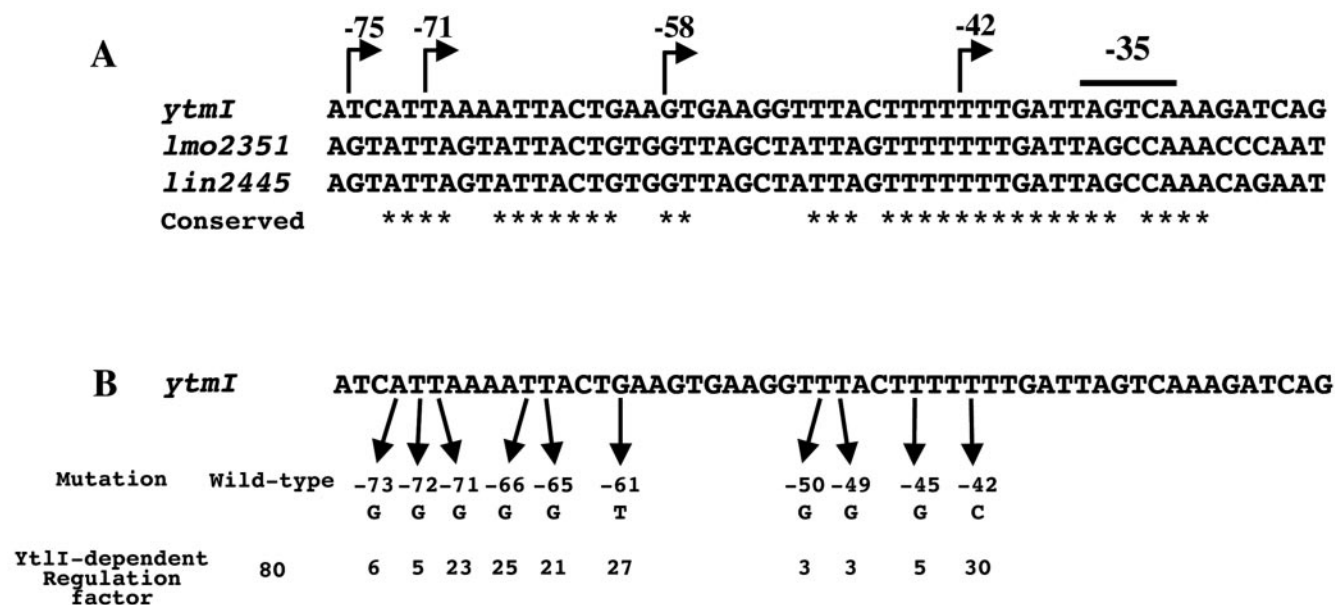
FIG. 4. Site-directed mutagenesis in a conserved motif present upstream from the promoter regions of the *ytmI*, lmo2351, and lin2445 operons. A: Conserved nucleotides in the three promoter regions are indicated by stars. Deletion end points of the different *ytmI'-lacZ* fusions are indicated by broken arrows. B: Mutations in the *cis*-acting target of the *ytmI* promoter region are indicated. For each point mutation, the YtlI-dependent regulation factor was obtained by calculating the ratio between the β-galactosidase activity of the fusion in a wild-type strain and in a *ytlI::aphA3* mutant after growth with methionine as sulfur source.

the *ytmI* expression, which was repressed with sulfate in BSIP1762 (pΔE*ytmI-lacZ*), was constitutive in strain BSIP1783 (pΔE*ytmI'-lacZ ytlI::aphA3 thrC::pxylA-ytlI*). The introduction of p*xylA*-lmo2352 instead of p*xylA-ytlI* into BSIP1764 also led to constitutive *ytmI* expression (Table 2, line 7). In the conditions tested, the transcriptional activation of *ytmI* by YtlI or Lmo2352 in *B. subtilis* did not involve modulation of their activity due to the presence of methionine or sulfate.

Identification of a sequence necessary for sulfur-dependent regulation of the *ytlI* gene. The *ytlI* expression was high in the presence of methionine and reduced with sulfate (1). In a *ytlI* mutant, the sulfur-dependent regulation of *ytlI* was conserved, indicating a YtlI-independent mechanism of control of *ytlI* expression (data not shown). To identify the DNA region necessary for the sulfur-mediated control, *ytlI* promoter regions containing various 5′ or 3′ deletions were fused to the *lacZ* gene (Fig. 1). These fusions were introduced as a single copy at the *amyE* locus of *B. subtilis* 168. β-Galactosidase activity was measured in the different strains grown in minimal medium with methionine or sulfate as sole sulfur source. The pΔI(−25 to +111) fusion gave very low β-galactosidase activity in the presence of either methionine or sulfate (Table 3, line 4). Deletion of the mapped promoter abolished *ytlI* transcription, confirming the existence of a single promoter. The pΔF(−130 to +111), pΔJ(−130 to −1), and pΔG(−46 to +111)*ytlI'-lacZ* fusions were 10- to 35-fold more expressed with methionine than with sulfate (Table 3, lines 1 to 2 and 5). In contrast, the pΔH(−39 to +111)*ytlI'-lacZ* fusion was constitutively expressed (Table 3, line 3). These results strongly suggested that the DNA region located between positions −46 and −39 upstream from the transcriptional start site was necessary to observe a repression by sulfate of *ytlI* transcription.

To confirm the role of the DNA sequence located upstream from the −35 box in the regulation of the *ytlI* gene, point mutations were introduced in this region using site-directed mutagenesis. The pΔF*ytlI'-lacZ* fusions containing various mutations were inserted at the *amyE* locus of the wild-type strain. The level of β-galactosidase activity in the corresponding mutants was determined after growth with methionine or sulfate (Table 3, lines 6 to 13). The modification of the A nucleotide at position −45 or the T at position −38 in the *ytlI* promoter region led to constitutive expression of the corresponding fusion, while a pΔF*ytlI'-lacZ* fusion containing a replacement of a C by an A in position −42 was still twofold repressed in the presence of sulfate. For the mutations C-41→G and A-39→G, the expression of the *ytlI'-lacZ* fusion increased in the presence of sulfate and decreased in the presence of methionine alone compared to the wild-type fusion. The level of expression with sulfate for fusions containing modifications at position −46 and −43 was similar to the wild-type strain, while the level of expression with methionine decreased. These results confirmed that the region located between positions −45 and −38 was important for the regulation of expression of the *ytlI* gene in response to sulfur availability. However, the intermediate level of expression observed with several mutations in the presence of methionine suggested that the sulfur-dependent regulation of the *ytlI* gene was probably complex.

YrzC is a repressor controlling the *ytlI* expression. Solovieva et al. (42) have recently characterized mutations leading to an increase of transcription of the *ribR* gene, which belongs to the *ytmI* operon and encodes a riboflavine kinase (41). Mutations are located either in the ribosome-binding site or in the coding sequence (substitution of a Ser for an Asn at position 58) of the *yrzC* gene (42). YrzC acts as a negative regulator of the *ytmI*
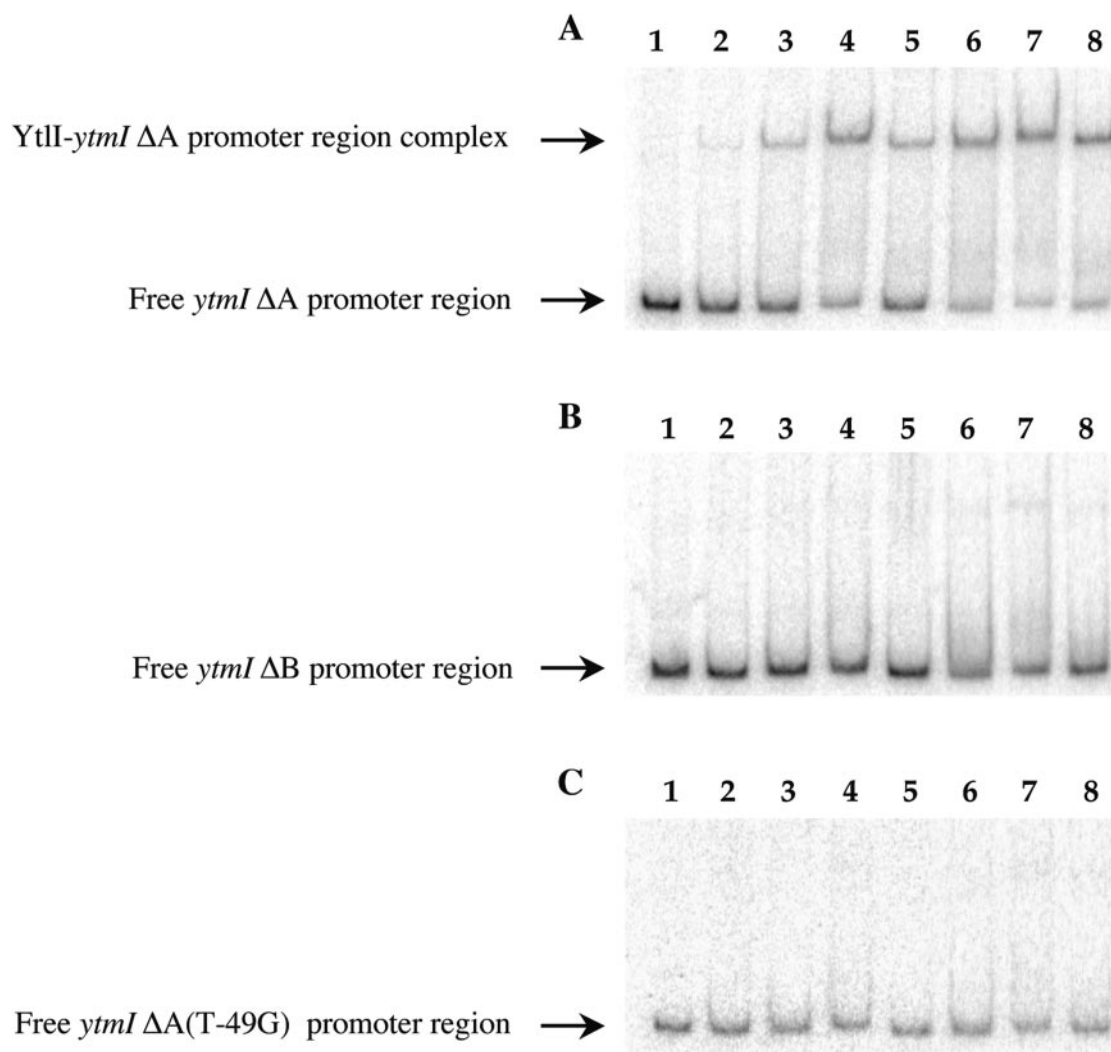
FIG. 5. Binding of the YtlI regulator to a *ytmI* promoter region fragment in a mobility shift assay. Gel mobility shift experiments were performed by incubating YtlI with 5′-radiolabeled DNA fragments containing different *ytmI* promoter regions: $\Delta A(-75$ to $+47)$ (A), $\Delta B(-71$ to $+47)$ (B), or $\Delta A(T-49 \rightarrow G)$ (C) DNA fragment. Lane 1, free probe; lanes 2 to 8, increasing amounts of YtlI-His$_6$ (12.5, 25, 50, 100, 200, 400, and 800 ng, respectively).

operon. To analyze the role of YtlI and YrzC in the control of *ytmI* expression, we have obtained a strain BSIP1797 containing an in-frame deletion of the *yrzC* gene and a pΔA*ytmI'*-*lacZ* fusion by double transformation of the wild-type *B. subtilis* strain (see Materials and Methods). Strain BSIP1811 ($\Delta yrzC$ *ytlI*::*aphA3* pΔA*ytmI'*-*lacZ*) was then constructed by transforming strain BSIP1797 with the chromosomal DNA of BSIP1214 (*ytlI*::*aphA3*) (Table 1). The β-galactosidase activity was assayed after growth of strains BSIP1256, BSIP1797, and BSIP1811 in minimal medium with methionine or sulfate as sole sulfur source (Table 2, lines 8 to 9). The introduction of a deletion in the *yrzC* gene led to constitutive expression of the pΔA*ytmI'*-*lacZ* fusion. This result was in agreement with the up-expression of the *ytmI* operon observed in a $\Delta yrzC$ mutant by Solovieva et al. (42). In a $\Delta yrzC$ *ytlI*::*aphA3* double mutant, the level of expression of the pΔA*ytmI'*-*lacZ* fusion was strongly reduced compared to that observed in a $\Delta yrzC$ mutant (Table 2, line 9). An epistatic effect of the *ytlI* mutation over

the *yrzC* deletion was observed. This strongly suggested that the role of YrzC on *ytmI* expression was indirect and mediated by YtlI. In addition, the expression of a pΔA*ytmI'*-*lacZ* fusion was no more repressed in the presence of sulfate in a $\Delta yrzC$ *ytlI*::*aphA3* double mutant while a residual repression was observed in a *ytlI* mutant (Table 2, lines 8 to 9). The YrzC regulator could therefore be involved in *ytmI* regulation via the second minor site of sulfur-dependent regulation proposed before.

The possible involvement of YrzC in the control of *ytlI* expression was also explored. To this purpose, a strain containing both a *yrzC* deletion and a pΔF*ytlI'*-*lacZ* fusion was constructed (see Materials and Methods). The expression of this fusion in a *yrzC*$^+$ and in a $\Delta yrzC$ background was measured after growth of the corresponding strains in the presence of methionine or sulfate (Table 3, lines 14 to 15). The pΔF*ytlI'*-*lacZ* fusion, which was repressed by sulfate in a wild-type strain, was constitutively expressed in a $\Delta yrzC$ mutant. This

TABLE 3. Effect of the sulfur source and of the YrzC regulator on the expression of different *ytlI'-lacZ* transcriptional fusions[a]

Strain	Relevant genotype	β-Galactosidase activity (nmol ONP min^{-1} mg of protein^{-1})	
		Methionine	Sulfate
BSIP1215	pΔF(−130 to +111)*ytlI'-lacZ*	215	6
BSIP1259	pΔG(−46 to +111)*ytlI'-lacZ*	43	4
BSIP1590	pΔH(−39 to +111)*ytlI'-lacZ*	25	50
BSIP1264	pΔI(−25 to +111)*ytlI'-lacZ*	2	3
BSIP1261	pΔJ(−130 to −1)*ytlI'-lacZ*	165	4.5
BSIP1728	pΔF(−46 A→C)*ytlI'-lacZ*	60	8
BSIP1714	pΔF(−45 A→C)*ytlI'-lacZ*	78	71
BSIP1720	pΔF(−43 T→C)*ytlI'-lacZ*	68	7
BSIP1729	pΔF(−42 C→A)*ytlI'-lacZ*	150	67
BSIP1721	pΔF(−41 C→G)*ytlI'-lacZ*	128	22
BSIP1730	pΔF(−40 T→A)*ytlI'-lacZ*	52	3
BSIP1715	pΔF(−39 A→G)*ytlI'-lacZ*	105	22
BSIP1716	pΔF(−38 T→A)*ytlI'-lacZ*	260	215
BSIP1215	pΔF(−130 to +111)*ytlI'-lacZ*	215	6
BSIP1793	pΔF(−130 to +111)*ytlI'-lacZ* ΔyrzC	118	98

[a] Cells were grown in minimal medium in the presence of sulfate or methionine at 1 mM final concentration. The β-galactosidase activities were obtained from cultures in mid-exponential growth phase. ONP, *O*-nitrophenol.

indicated that YrzC participated in the repression of *ytlI* expression.

The expression of lmo2352 is regulated by YrzC in *B. subtilis*. The activity of Lmo2352 as transcriptional activator does not seem to be modulated in response to sulfur availability in *B. subtilis*. This asked the question whether the synthesis of Lmo2352 was increased in the presence of methionine as observed for YtlI. A lmo2352'-*lacZ* fusion was then constructed and inserted at the *amyE* locus of *B. subtilis* strain 168. β-Galactosidase activity was 85 U (mg of protein)$^{-1}$ after growth with methionine and 4 U (mg of protein)$^{-1}$ after growth with sulfate. In *B. subtilis*, the transcription of lmo2352 was subject to a sulfur-dependent regulation. Since YrzC acts as a negative regulator of *ytlI* transcription, we tested the effect of a *yrzC* deletion on the expression of a lmo2352'-*lacZ* fusion. β-Galactosidase activity was 98 and 128 U (mg of protein)$^{-1}$ after growth with methionine or sulfate, respectively. A YrzC gene disruption also led to constitutive expression of lmo2352.

Expression of the *ytlI* gene in cysteine biosynthesis mutants. In order to get more insight into the nature of the effector(s) involved in the regulation of the *ytlI* gene, the expression of the pΔF*ytlI'-lacZ* fusion was tested in mutants deficient in cysteine biosynthesis. The pΔF*ytlI'-lacZ* fusion was introduced at the *amyE* locus of a *cysH*::Tn*10* mutant or a Δ*cysJI*::*aphA3* mutant lacking APS-PAPS reductase and sulfite reductase, respectively (3, 14, 25). The expression of the pΔF*ytlI'-lacZ* fusion was then tested after growth of the corresponding strains in the presence of 1 mM methionine, 1 mM methionine plus 1 mM sulfate, or 0.5 mM cysteine (Table 4). For strain BSIP1215, β-galactosidase activity was high after growth with methionine and reduced after growth with methionine plus sulfate or cysteine (Table 4, line 1). In a *cysH* or a Δ*cysJI* background, the expression of this fusion was similar in the presence of methionine or methionine plus sulfate. However, a repression was

TABLE 4. Expression of a pΔF *ytlI'-lacZ* fusion in cysteine biosynthesis mutants[a]

Relevant genotype	β-Galactosidase activity (nmol ONP min^{-1} mg^{-1})		
	Methionine	Methionine and sulfate	Cysteine
pΔF(−130 to +111)*ytlI'-lacZ*	215	4	4
pΔF(−130 to +111)*ytlI'-lacZ* *cysH*::Tn*10*	225	205	20
pΔF(−130 to +111)*ytlI'-lacZ* Δ*cysJI*::*aphA3*	229	205	7
pΔF(−130 to +111)*ytlI'-lacZ* *cysK*::*spc*	200	205	170

[a] Cells were grown in minimal medium in the presence of 1 mM methionine, 1 mM methionine and 1 mM sulfate, or 0.5 mM cysteine. The β-galactosidase activities were obtained from cultures in mid-exponential growth. The mean values of at least three independent experiments are presented. Standard deviations are less than 20% of the mean. ONP, *O*-nitrophenol.

still observed in the presence of cysteine (Table 4, lines 2 and 3). This suggested that sulfite or one of the intermediates in the sulfate reduction pathway from sulfate to sulfite did not play a major role in the regulation of the *ytlI* gene. The pΔF*ytlI'-lacZ* fusion was also introduced at the *amyE* locus of strain BSIP1304, in which the *cysK* gene, encoding OAS thiol-lyase is inactivated (2, 45). In the resulting strain, β-galactosidase activity was high in the presence of methionine, methionine plus sulfate, and cysteine (Table 4 line 4). In a Δ*cysK* mutant, neither sulfate nor cysteine repressed the expression of *ytlI*. The lack of OAS thiol-tyase probably led to higher OAS and sulfide concentrations and to a lower level of cysteine into the cell. We therefore tested the possible involvement of OAS in the regulation of the *ytlI* gene. The strain BSIP1215 containing the pΔF*ytlI'-lacZ* transcriptional fusion was grown in minimal medium in the presence of 0.5 mM cysteine until an optical density of 0.6 was reached. The culture was then separated into two samples: one containing 1 mM OAS, the second without OAS. β-Galactosidase was measured 1 h after the addition of OAS. The presence of OAS in the growth medium resulted in an increase in pΔF*ytlI'-lacZ* expression (86 U [mg of protein]$^{-1}$) compared to the level observed without OAS (24 U [mg of protein]$^{-1}$).

DISCUSSION

Several LysR-type regulators are involved in the control of sulfur metabolism in both gram-negative and gram-positive bacteria (9, 14, 16–18, 40, 49, 50). In this work, we characterized in more detail the role of the LysR-type regulator, YtlI, the transcriptional activator of the *B. subtilis* complex operon *ytmI* (5, 7, 41). The *ytlI* gene and the *ytmI* operon are transcribed divergently. The *ytlI* and *ytmI* promoters overlapped with the −10 boxes located at the same position (Fig. 2), an organization found for several LysR-like regulators (35). A *cis*-acting sequence important for the sulfur-dependent regulation of *ytmI* expression was identified using both deletion and site-directed mutagenesis. As observed for other members of this family of regulators (35), two regions seemed to be critical for the transcriptional control by YtlI: the AT at position −73/−72 and a second region around −50. The YtlI protein

was able to bind to the *ytmI* promoter region (−75 to +47) in gel shift experiments (Fig. 5). In contrast, the affinity of YtlI for the promoter region with the −75 to −71 DNA sequence deleted or modified in positions −49 or −73 strongly decreased. In silico analysis suggested that the YtlI-binding site was not found upstream from other *B. subtilis* sulfur-regulated genes in agreement with the absence of a *ytlI*-dependent control for most of these genes (1, 7) (S. Auger, unpublished results). This indicated that YtlI was probably not a global regulator of sulfate assimilation, cysteine biosynthesis, and cystine transport in *B. subtilis*, in contrast to the CysB regulator from *E. coli*. In addition, neither the key residues important for CysB activity nor the CysB-binding sites are conserved for YtlI (18, 22). This activator shares only weak similarities (less than 30%) with the other LysR-type regulators involved in the control of sulfur metabolism including CysB but also CmbR and MtaR from *Lactococcus lactis* and group B streptococci (9, 11, 40). This indicates the existence of differences in sulfur-dependent regulation in these microorganisms.

The expression of the *ytlI* gene itself was regulated in response to sulfur availability independently of YtlI, indicating that another regulator was involved. A *cis*-acting target important for the sulfur-dependent regulation of *ytlI* expression was identified just upstream from the −35 box of this gene (Fig. 3B and Table 3). The deletion or some point modifications of this sequence led to constitutive expression of *ytlI*, suggesting that a repressor was probably required in this control. The YrzC regulator, which was recently identified as a negative regulator of the *ytmI* operon (42), was a good candidate to play this role. In this work, we showed that an in-frame deletion of *yrzC* led to a high level of expression of a *ytmI-lacZ* or a *ytlI-lacZ* fusion after growth with either sulfate or methionine. In addition, the presence of the YtlI activator was necessary to observe constitutive expression of *ytmI* expression in a *yrzC* mutant (Table 2, line 9). Our genetic analyses strongly suggested that the YrzC repressor mediated sulfur-dependent regulation of this operon by controlling the synthesis of the YtlI activator. The direct or indirect effect of YrzC on *ytlI* transcription remains to be determined.

A large operon encoding an L-cystine ABC transporter (5), two putative monooxygenases, a putative acetyltransferase (YtmI), and a protein sharing weak similarities with glutaredoxine (YtnI) is present in *B. subtilis* and in *Listeria* spp. The high level of conservation between the *ytmI*, lmo2351, and lin2445 operons suggested a common function, the uptake of L-cystine and related products by the ABC transporter and the degradation of one or several sulfur compounds (5, 39). *B. subtilis* and *Listeria* spp. are usually present in soils or in contact with plants (32). These conserved operons could be involved in the assimilation of sulfur compounds present in a common biotope. The lmo2352 and lin2446 genes, adjacent to the lmo2351 and lin2445 operons, encode regulators highly similar to the YtlI activator. Several data strongly suggested a conserved cascade of regulation for the *ytmI*-type operons in *B. subtilis* and *Listeria*: (i) the Lmo2352 regulator was able to replace the YtlI protein to activate *ytmI* transcription; (ii) a conserved motif was found upstream from the −35 box of the *ytmI*, lmo2351, and lin2445 operons (Fig. 4A) in the region important for the YtlI-dependent regulation of *ytmI*; (iii) a

sulfur-dependent regulation of the lmo2352 gene was observed in *B. subtilis*; (iv) YrzC was a negative regulator of lmo2352 expression in this bacterium. In addition, YrzC shares 69% identity with Lmo1515 and Lin1550, which probably play the same role as this repressor in *Listeria*. A conserved sequence, AT(A/T)ATTCCTAT, was found in the promoter regions of *ytlI*, lmo2352, and lin2446 (Fig. 3B). The YrzC repressor or another regulator could bind to this motif.

The uptake of L-cystine by TcyJKLMN in *B. subtilis* and the assimilation of taurine and sulfonate in *E. coli* are controlled by a cascade of regulation. In the latter, the CysB activator controls the expression of a second regulatory gene *cbl* by binding to its promoter region (16). In addition, both CysB and Cbl directly bind to the *ssu* and *tau* promoter regions to modulate their expression in response to cysteine and sulfate availability (6, 47, 48). In the same way, the organization of the *ytmI* promoter region is complex, with the existence of two *cis*-acting targets: the YtlI-binding site present upstream from the −35 box and a second minor site located downstream from the promoter, which could correspond to a direct or indirect target for YrzC (Fig. 2). A major difference was observed for the YtlI-dependent regulation compared to Cbl- or CysB-dependent regulation. Like most of the LysR-type regulators, an effector (APS or *N*-acetylserine) modulates the transcriptional activation by Cbl or CysB (6, 22). In contrast, our results suggested that the repression by sulfate controlled only the synthesis of YtlI and not its activity. Similarly, the nitrogen assimilation control regulator, NAC, of *Klebsiella aerogenes* activates the trancription of the *hut* operon or represses *gdh* expression independent of the nitrogen supply (36). The expression of the *nac* gene itself is under the control of the NTR system involving transcriptional activation of the *nac* gene by the σ^{54}-dependent regulator NtrC (8).

In addition to the regulation of *ytlI* transcription by the YrzC repressor, we also unraveled some features of the control of *ytlI* expression in response to sulfur availability. As observed for the *cysH* and *ssu* operons involved in sulfate or sulfonate assimilation (23, 44), the addition of OAS to the culture medium led to a partial derepression of *ytlI* transcription. In addition, constitutive expression of this gene was obtained in a *cysK* mutant (inactivated for OAS thiol-lyase), which could accumulate OAS. These results suggested a role for OAS in the signaling pathway controlling *ytlI*, *ssu*, and *cysH* expression. Mansilla et al. (23) have proposed that the *cysH* operon is regulated by the binding of an unknown repressor, CysR, to a sequence located upstream of the −35 box. Despite the absence of a common motif in the *cysH* and *ytlI* promoter regions, the possibility that YrzC participated in *cysH* regulation remains to be seen.

OAS is synthesized by the serine transacetylase, the *cysE* gene product. In *B. subtilis*, *cysE* expression is regulated by transcription antitermination at a cysteine-specific T-box (10). The level of OAS is therefore correlated to the level of uncharged cysteinyl-tRNA, which signals the cysteine status in the cell. YrzC shares 31% identity with IscR, the repressor of the *iscRSUA* operon of *E. coli* involved in Fe-S cluster biogenesis (37). [2Fe-2S]-IscR is the active form of the repressor. Interestingly, the [2Fe-2S] cluster may be coordinated by three cysteine residues of IscR (37), which are absent in YrzC. The

binding of a [2Fe-2S] cluster to YrzC seems therefore unlikely while its interaction with an effector like OAS could be involved.

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